

10/644123

CofC

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REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 CFR 1.322  
Docket No. UF-314XC1  
Patent No. 6,929,940

Doran R. Pace, Patent Attorney

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Nigel Gordon John Richards, Christopher Harry Chang, Ammon B. Peck  
Issued : August 16, 2005  
Patent No. : 6,929,940  
For : Polynucleotides Encoding Oxalate Decarboxylase from *Aspergillus Niger* and Methods of Use

Mail Stop Certificate of Corrections Branch  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**Certificate**  
NOV 10 2005  
**of Correction**

REQUEST FOR CERTIFICATE OF CORRECTION  
UNDER 37 CFR 1.322 (OFFICE MISTAKE)

Sir:

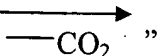
A Certificate of Correction (in duplicate) for the above-identified patent has been prepared and is attached hereto.

In the left-hand column below is the column and line number where errors occurred in the patent. In the right-hand column is the page and line number in the application where the correct information appears.

**Patent Reads:**

Column 2, line 50  
"of OXDC"

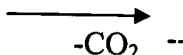
Column 2, Scheme 1 (A)

""

**Application Reads:**

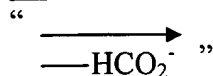
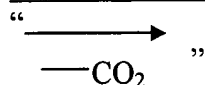
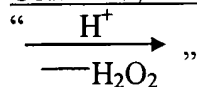
Page 3, line 4  
-- of OxDC --

Page 3, line 5, Scheme 1 (A)

--  --

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**Patent Reads:**Column 3, Scheme 1 (A)Column 3, Scheme 1 (B)Column 3, Scheme 1 (B)Column 3, Scheme 1 (legend)

"and formulate via"

Column 3, Scheme 1 (legend)"manganese ion (Mn<sup>+</sup>)"Column 6, line 4

"also showing bold"

Column 6, line 11"*subtilus*"Column 10, line 53

"for OXDC"

Column 10, line 66

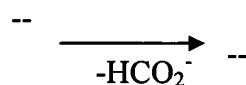
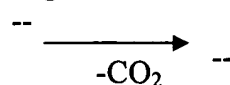
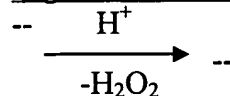
"Native OXDC"

Column 11, line 53"*Aspergillus niger* OXDC was"Column 13, line 6

"OXDC exhibited"

Column 13, line 35

"two "His-TrpHis" motifs"

**Application Reads:**Page 3, line 5, Scheme 1 (A)Page 3, line 5, Scheme 1 (B)Page 3, line 5, Scheme 1 (B)Page 3, Scheme 1 (legend)

-- and formate via --

Page 3, Scheme 1 (legend)-- manganese ion (M<sup>n+</sup>) --Page 7, line 9

-- also shown in bold --

Page 7, line 13-- *subtilis* --Page 14, line 18

-- for OxDC --

Page 14, line 26

-- Native OxDC --

Page 16, line 3-- *Aspergillus niger* OxDC was --Page 17, line 22

-- OxDC exhibited --

Page 18, lines 16-17

-- two "His-TrpHis" motifs --

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**Patent Reads:**

Column 13, line 38  
"OXDC suggests"

Column 15, line 17  
"100 mL imidazole HCl buffer"

Column 15, line 24  
"200 ml imidazole.HCl buffer"

Column 15, line 38  
"hexamethylenetetramine.HCl pH 7."

Column 16, line 23  
"arid some"

Column 16, line 63  
"Azarn, M.,"

Column 18, line 30  
"and Bomemann, S."

**Application Reads:**

Page 18, line 18  
-- OxDC suggests --

Page 20, lines 26-27  
-- 100 mL imidazole·HCl buffer --

Page 21, lines 1-2  
-- 200 mL imidazole·HCl buffer --

Page 21, line 10  
-- hexamethylenetetramine·HCl pH 7. --

Page 23, line 1  
-- and some --


Page 23, line 41  
-- Azam, M. --

Page 25, line 37  
-- and Bornemann, S. --

A true and correct copy of pages 3, 7, 14, 16-18, 20, 21, 23, and 25 of the specification as filed which support Applicants' assertion of the errors on the part of the Patent Office accompanies this Certificate of Correction.

Approval of the Certificate of Correction is respectfully requested.

Respectfully submitted,



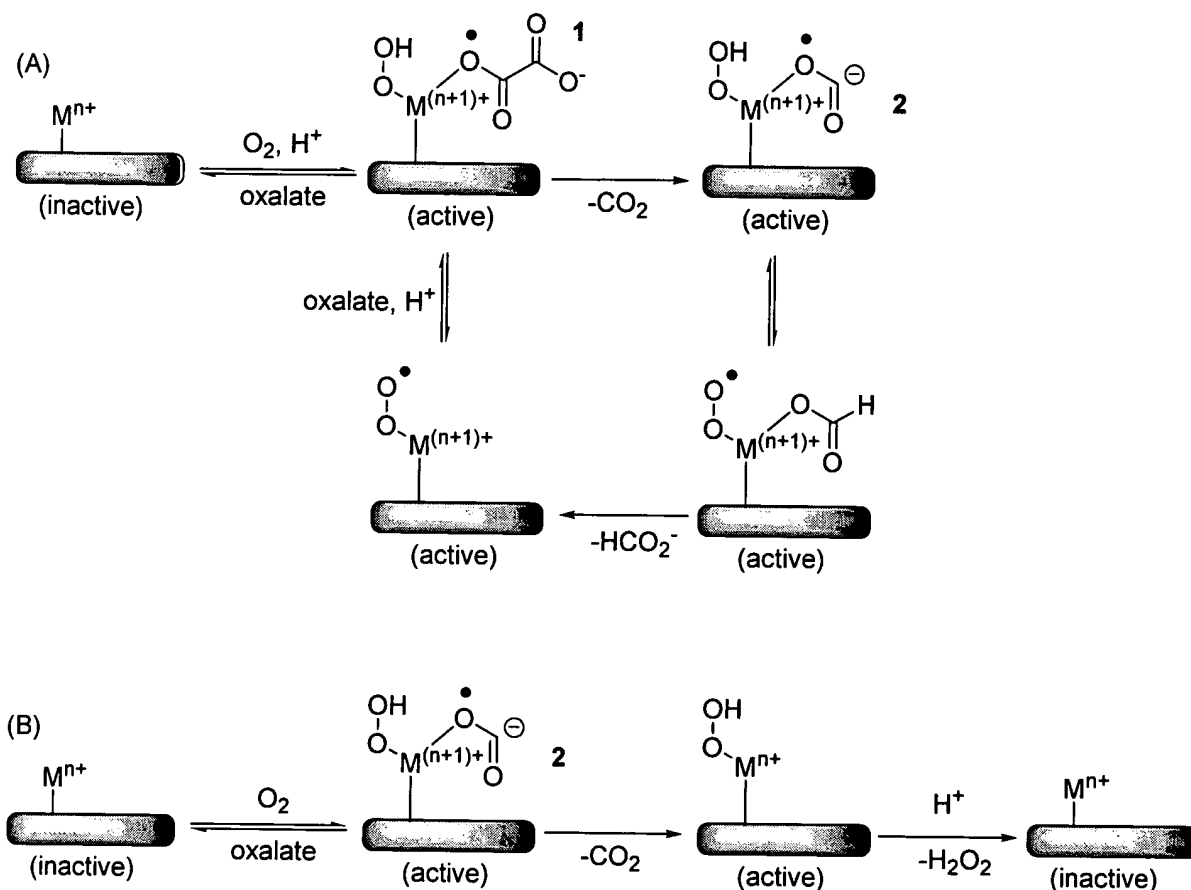
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DRP/ehm

Attachments: Copy of pages 3, 7, 14, 16-18, 20, 21, 23, and 25 of the specification

the two enzymes (Dunwell *et al.*, 2000). The observed correlation between  $\text{H}_2\text{O}_2$  formation and  $\text{pO}_2$  in the OxDC-catalyzed reaction (Emiliani *et al.*, 1968) is consistent with such a mechanism if oxidation of the formyl radical anion **2** takes place to generate  $\text{CO}_2$ , peroxide anion and an inactive form of OxDC (Scheme 1B).

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**Scheme 1.** (A) Hypothetical mechanism for OxDC-catalyzed conversion of oxalate into  $\text{CO}_2$  and formate via homolytic C-C bond cleavage. Note that the interaction of the manganese ion ( $\text{M}^{n+}$ ) with oxygen and oxalate remains to be established experimentally. (B) Side reaction proposed to consume oxygen during turnover.

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Although it has been speculated that Mn(III) and Mn(IV) are the redox active forms of the metal during catalysis (Anand *et al.*, 2002), there is no published evidence to support such a claim. Equally, the intermediacy of a protein-based radical cannot be ruled out on the basis of current biochemical and structural information on *Bacillus subtilis* OxDC. This proposal has the merit of rationalizing the observed correlation between the amounts of hydrogen peroxide

**Figure 2** shows the deduced primary structure of *Aspergillus niger* oxalate decarboxylase protein from the cDNA sequence shown in Figure 3. Amino acids that define the signal peptide of the protein are shown in italic font. Standard one letter code is used to represent amino acids.

**Figure 3A-C** shows the alignment of the nucleotide sequences of the gene encoding oxalate decarboxylase *Aspergillus niger* (genomic) OxDC and the cDNA obtained from mRNA isolated from the fungus (cDNA). Underlined residues in the genomic sequence indicate the location of the two introns deduced to be present in the gene by comparison of the sequences. These are both flanked by canonical sequences shown in bold typeface. The TAG sequence at the 3'-end of the gene, also shown in bold typeface, indicates the end of the region coding for the protein product.

**Figure 4** shows the DNA sequence encoding oxalate decarboxylase as cloned from genomic DNA of *Aspergillus niger*.

**Figure 5** shows the deduced primary structure of *Bacillus subtilis* yvrk protein. Standard one letter code is used to represent amino acids.

#### Brief Description of Sequences

**SEQ ID NO. 1** is a genomic polynucleotide of *Aspergillus niger* encoding an oxalate decarboxylase enzyme that can be used according to the present invention.

**SEQ ID NO. 2** is a cDNA sequence of *Aspergillus niger* encoding an oxalate decarboxylase enzyme that can be used according to the present invention.

**SEQ ID NO. 3** is the amino acid sequence of an oxalate decarboxylase enzyme of *Aspergillus niger* encoded by SEQ ID NO. 1.

**SEQ ID NO. 4** is an amino acid sequence of an oxalate decarboxylase enzyme of the invention with the amino acid leader sequence removed.

**SEQ ID NO. 5** is a sequence of a PCR primer that can be used according to the present invention.

**SEQ ID NO. 6** is a sequence of a PCR primer that can be used according to the present invention.

**SEQ ID NO. 7** is a partial sequence of the oxalate decarboxylase enzyme of the present invention.

Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.*, 1983):

$T_m = 81.5^\circ\text{C} + 16.6 \log[\text{Na}^+] + 0.41(\%G+C) - 0.61(\% \text{ formamide}) - 600/\text{length of duplex in base pairs.}$

5 Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at  $T_m - 20^\circ\text{C}$  for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

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All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

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### Materials and Methods

Culture Conditions. *Aspergillus niger* (ATCC 26550) was maintained on potato dextrose agar plates at  $4^\circ\text{C}$ . Inoculating a starter culture of ATCC medium 950 with a loopful of *Aspergillus niger* spores produced mycelium for OxDC purification. The literature procedures for inducing OxDC production were modified by the substitution of sucrose for glucose (by weight),  $\text{NH}_4\text{Cl}$  for  $\text{NaNO}_3$  (by molarity) and the addition of 10 mM sodium oxalate (Sigma). After growth at  $30^\circ\text{C}$  for several days, this starter culture was used to inoculate larger cultures. Fungus for DNA isolation was grown on yeast extract-peptone-dextrose medium (ATCC medium 1005), with cultures being shaken at  $37^\circ\text{C}$  until the mycelium was confluent. Mycelium was harvested by vacuum filtration, washed with de-ionized water, frozen in powdered dry ice, lyophilized, and stored at  $-80^\circ\text{C}$  until used in subsequent experiments.

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Isolation of Native OxDC. Freeze-dried mycelium was ground in a mortar and pestle with powdered dry ice. After sublimation, the powder was suspended in 20 mM NaOAc pH 5.6 buffer plus 0.1% Tween 20. Insoluble material was removed by centrifugation, and methanol added to the supernatant to a final concentration of 50% v/v. The resulting mixture was incubated at  $0^\circ\text{C}$  for 30 minutes before collection of precipitated material by centrifugation. The

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37 °C for 30 minutes. Formate was quantitated by comparison to a standard curve generated by spiking protein-free OxDC assays with known amounts of sodium formate.

Metal Analysis of *Aspergillus niger* OxDC. Purified *Aspergillus niger* OxDC was treated to remove adventitious, surface-bound metals by incubating 9 mg/mL OxDC (100 µL) with 10 mM *o*-phenanthroline (10 µL) on ice for 30 minutes. The enzyme was then desalted over G25 Sephadex resin that had been pre-treated with 20 mM NaOAc, pH 5.2, containing 2 mM EDTA and then equilibrated with Chelex-100-treated 20 mM NaOAc buffer, pH 5.2. In these experiments, all glassware was washed with 1 M HNO<sub>3</sub> and rinsed with deionized water (18.3 M) to remove exchangeable metal ions prior to use. Samples of OxDC treated in this manner were then divided into two aliquots for EPR and inductively coupled plasma-atomic emission (ICP-AE) spectroscopy. Metal content was determined by ICP-AE spectroscopy using protein samples made by diluting 100 µL of OxDC (0.5 mg) with 9.9 mL deionized water. All analyses were performed in the Department of Chemistry at the University of Florida. Calculations of the metal content in native *Aspergillus niger* OxDC employed standard procedures (see supplementary material).

Chromosomal DNA Cloning. Freeze-dried mycelium (0.5 g) taken from shake cultures of confluent *Aspergillus niger* was gently ground in liquid N<sub>2</sub>, using a mortar and pestle, to give a fine powder. Care was taken during this procedure so as to prevent shearing high-molecular weight DNA by excessive grinding. The resulting powder was extracted with Qiagen "QBT" buffer (20 mL) supplemented with 0.5% v/v Triton X-100, before the addition of solutions of ribonuclease A (100 µL) and 14 mg/mL Proteinase K (100 µL). The extract was incubated for 30 min at ambient temperature, and then for 15 min at 50 °C before being loaded onto a Qiagen Genomic Tip and purified. The resulting high molecular weight DNA was digested thoroughly using *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I restriction enzymes.

Polymerase chain reaction (PCR) primers were designed assuming a close nucleotide sequence relationship between the genes encoding OxDC in *Aspergillus phoenices* (Scelonge *et al.*, 1998) and *Aspergillus niger*. A mutagenic 5'-primer (5'-GTCCTCGAGAAAAGATACCAG-3') (SEQ ID NO. 5) was employed to introduce a *Xho*I site and a proteolytic cleavage site, for use in future expression experiments, immediately upstream of the codon of Tyr-24 in the putative *Aspergillus niger* gene sequence. This primer was

combined with a reverse primer (5'-TCATCTACTCACTTGGGCTCCGAATTG -3') (SEQ ID NO. 6) matching the 3'-end of the gene in *Aspergillus phoenices*. Thirty cycles of amplification were performed (95 °C, 1' denaturation; 45 °C primer annealing, 30 s; 74 °C primer extension, 3') with *Pfu* polymerase (Promega, Madison, WI), and the resulting PCR product purified by  
5 phenol:chloroform:isoamyl alcohol extraction, chloroform extraction, and ethanol precipitation. The plasmid pPIC9K was digested with *Sna*BI, treated with alkaline phosphatase, and purified prior to overnight ligation with the purified PCR product. Competent JM109 cells (>10<sup>8</sup> CFU/μg) were transformed, and white colonies screened by *Xho*I digestion of alkaline lysis/miniprep plasmid DNA. Plasmid that produced two bands upon *Xho*I digestion was  
10 purified, and submitted for nucleotide sequencing at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Isolation, Purification, and Assay of Native Oxalate Decarboxylase from *Aspergillus niger*.

Previous studies had shown that the presence of OxDC in the mycelium of *Aspergillus niger* was inversely related to detectable oxalate in the extracellular milieu (Emiliani *et al.*, 1964). This might be rationalized by assuming that there is leakage of protonated oxalic acid  
15 back into the fungus when the pH of the culture drops due to excretion of oxalic and citric acids during the early stages of *Aspergillus* growth. As a consequence, OxDC expression is induced so as to reduce oxalate concentrations in the mycelium to a non-toxic level. In light of this hypothesis, literature protocols for inducing OxDC production in *Aspergillus niger* were modified by adding 10 mM sodium oxalate to the minimal media upon which the fungus was  
20 grown. Under these conditions, adequate amounts of enzyme could be isolated from the fungal mycelium for the work described here. Previous studies had demonstrated that native *Aspergillus niger* OxDC exhibited notable stability in organic solvents, and so initial purification steps involved precipitation of the enzyme with methanol. Subsequent chromatography using



anion exchange and hydrophobic interaction columns gave OxDC as a single band on SDS-PAGE (Figure 1), with a molecular weight in the range expected based on studies of the enzyme isolated from *Flammulina velutipes* (Kathiara *et al.*, 2000). Purified *Aspergillus niger* OxDC exhibited a specific activity of 10 I.U./mg, as determined from steady-state formate production under initial velocity conditions.

#### Example 2 - Deduced Primary Structure of *Aspergillus niger* OxDC.

Cloning of both the chromosomal and cDNA copies of the OxDC gene showed that (i) there are only two intron sequences in the *Aspergillus niger* decarboxylase gene (Figure 3A-3C), in contrast to the 17 reported for the cognate gene in *Flammulina velutipes* (Kesarwani *et al.*, 2000), and (ii) the intron sequences in the gene encoding OxDC in *Aspergillus niger* have canonical 5' and 3' borders (GT-AG) in contrast to those reported to be present in the cognate gene in *Aspergillus phoenices* (Scelonge *et al.*, 1998). The protein product encoded by the *yvrk* gene in *Bacillus subtilis* (Kunst *et al.*, 1997) shows some homology to *Aspergillus niger* OxDC with 197 (52%) residues in the bacterial OxDC being identical to those in the fungal enzyme.

More importantly for the catalytic mechanism of oxalate degradation, there are two “His-Trp-His” motifs that are conserved among the bacterial and fungal oxalate decarboxylases. Recent work on recombinant *Bacillus subtilis* OxDC suggests that this enzyme contains Mn(II) in its resting state (Tanner *et al.*, 2001; Anand *et al.*, 2002) consistent with our observations on the native *Aspergillus niger* OxDC. In addition, a similar “His-Ile-His” motif present in oxalate oxidase has been shown to be a manganese-binding site by X-ray crystallography (Woo *et al.*, 2000), suggesting a role for at least one, and possibly two metal ions, in OxDC catalysis (Gane *et al.*, 1998).

#### Example 3 - Biochemical Characterization of Native *Aspergillus niger* OxDC.

With the successful development of culture conditions and purification procedures to obtain milligram amounts of native *Aspergillus niger* OxDC, the biochemical and spectroscopic properties of the enzyme were investigated. N-terminal sequencing of the purified protein, carried out at the Protein Core Facility of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida (UF), revealed that phenylalanine is the first

sequence of the gene in the GenBank database, in order to clone the bacterial gene, and express and characterize its encoded protein. These primers were such that the *yvrK* coding sequence would be in-frame with the T7 control elements that are part of the pET-9a expression vector (Stratagene). An *NdeI* site was included at the N-terminal methionine, and a *BamHI* site after the termination codon of *yvrK*. *B. subtilis* 168 genomic DNA was purified from an overnight 5 mL culture using a Genomic DNA Miniprep kit (Qiagen). The *yvrK* sequence was amplified for 31 cycles (95 °C denaturation, 30s; 45 °C annealing, 30 s; 74 °C extension, 2 min). The resulting DNA was digested with *NdeI* and *BamHI*, then ligated into pET-9a digested similarly. Competent JM109 cells were transformed with the ligation mixture and with pET-9a as a control, and transformants selected on Luria-Bertani broth (LB) containing 30 µg/mL kanamycin (LBK). The resulting colonies were screened by *NdeI*-*BamHI* digestion to confirm the presence of a ~1153 bp insert, and the sequence of the cloned gene was checked by sequencing. A plasmid produced from pET-9a/*yvrK*:JM109 by standard alkaline lysis miniprep was used to transform the expression strain BL21(DE3), and the expression of the *Yvrk*-encoded protein was tested by inoculating 0.5 L of LBK supplemented with pET-9a/*yvrK*:BL21(DE3). The cells were grown at 37 °C and shaken at 200 r.p.m. When the cultures reached  $A_{600}$  of 2 they were heat shocked in water bath at 42 °C for 18 min before the addition of isopropyl thiogalactoside (IPTG) and  $MnCl_2$  to final concentrations 1 and 5 mM respectively. The cells were harvested after 4 h of shaking by centrifugation (5,000 x g, 15 min, 4 °C). Pellets were resuspended in 50 mL lysis buffer (50 mM Tris/HCl pH 7; 10 µM  $MnCl_2$ ) and sonicated for 30 s at 80 % power. After sonication, lysis pellets were separated from the crude extract by centrifugation (8000 rpm, 20 min, 4°C) and resuspended in 50 mL of extraction buffer containing 1 M sodium chloride, 0.1% Triton X-100, and 10 mM 2-mercaptoethanol. The mixture was stirred overnight at room temperature. Cell debris was removed by centrifugation and the supernatant was combined with the crude extract. This solution (100 mL) was diluted 10-fold before it was applied to a 2.5 x 30 cm DEAE-Sepharose Fast Flow (Sigma) column. This column was washed with 100 mL imidazole-HCl buffer (20 mM; pH 7.0 and 10 µM  $MnCl_2$ ) and developed with a 500 mL M NaCl gradient (0 to 1M gradient). Ten mL fractions were collected and assayed for their ability to oxidize *o*-phenylenediamine, which is a side-reaction catalyzed by OxDC. Fractions exhibiting activity were pooled and solid  $(NH_4)_2SO_4$  was added to a 70 % saturation. The precipitate was

removed by centrifugation (8,000 x g, 30 min, 4 °C) and redissolved in 200 ml imidazole-HCl buffer and the supernatant was loaded onto a phenyl-Sepharose Hi-Performance (Amersham Pharmacia Biotech) column. The column was washed with imidazole hydrochloride buffer (50 mM, pH 7.0, containing 10  $\mu$ M MnCl<sub>2</sub>) and developed with a 500 mL (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient (1.7 to 0 M). The fractions were pooled as for the DEAE column and diluted 15-fold before they were loaded onto a Q-Sepharose Hi-Performance (Amersham Pharmacia Biotech) column. The protein was eluted with an imidazole hydrochloride buffer (50 mM, pH 7.0, containing 10  $\mu$ M MnCl<sub>2</sub>) and a 500 mL NaCl gradient (0 to 1 M) as for the DEAE column. Protein precipitated with 70 % ammonium sulfate was centrifuged and redissolved in 10 ml 20 mM hexamethylenetetramine-HCl pH 7. Ammonium sulfate was dialyzed out against 1L of the same amine buffer for 5 h at 4 °C. Protein solution was concentrated by centrifugal concentrator to final volume ~ 1 ml. Aliquots were flash-frozen in liquid nitrogen and stored at -80 °C. This procedure gave highly purified OxDC in yields of up to 30-40 mg/L with a specific activity of approximately 50 IU/mg.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,929,940

Page 1 of 3

APPLICATION NO.: 10/644,123

DATED : August 16, 2005

INVENTORS : Nigel Gordon John Richards, Christopher Harry Chang, Ammon B. Peck

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 2, line 50

"of OXDC" should read -- of OxDC --.

Column 2, Scheme 1 (A)

" $\xrightarrow{\text{---CO}_2}$ " should read --  $\xrightarrow{\text{---CO}_2}$  --.

" $\xrightarrow{\text{---HCO}_2^-}$ " should read --  $\xrightarrow{\text{---HCO}_2^-}$  --.

Column 3, Scheme 1 (B)

" $\xrightarrow{\text{---CO}_2}$ " should read --  $\xrightarrow{\text{---CO}_2}$  --.

" $\xrightarrow{\text{---H}^+}$ " should read --  $\xrightarrow{\text{---H}^+}$  --.

Column 3, Scheme 1 (legend)

"and formulate via" should read -- and formate via --.

"manganese ion (Mn<sup>+</sup>)" should read -- manganese ion (M<sup>n+</sup>) --.

MAILING ADDRESS OF SENDER:

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NOV 14 2005

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,929,940

Page 2 of 3

APPLICATION NO.: 10/644,123

DATED : August 16, 2005

INVENTORS : Nigel Gordon John Richards, Christopher Harry Chang, Ammon B. Peck

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 6, line 4

“also showing bold” should read -- also shown in bold --.

Column 6, line 11

“*subtilus*” should read -- *subtilis* --.

Column 10, line 53

“for OXDC” should read -- for OxDC --.

Column 10, line 66

“Native OXDC” should read -- Native OxDC --.

Column 11, line 53

“*Aspergillus niger* OXDC was” should read -- *Aspergillus niger* OxDC was --.

Column 13, line 6

“OXDC exhibited” should read -- OxDC exhibited --.

Column 13, line 35

“two “His-TrpHis” motifs” should read -- two “His-Trp-His” motifs --.

Column 13, line 38

“OXDC suggests” should read -- OxDC suggests --.

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Column 15, line 17

“100 mL imidazole HCl buffer” should read -- 100 mL imidazole·HCl buffer --.

Column 15, line 24

“200 ml imidazole.HCl buffer” should read -- 200 mL imidazole·HCl buffer --.

Column 15, line 38

“hexamethylenetetramine.HCl pH 7.” should read -- hexamethylenetetramine·HCl pH 7. --.

Column 16, line 23

“arid some” should read -- and some --.

Column 16, line 63

“Azarn, M.” should read -- Azam, M. --.

Column 18, line 30

“and Bomemann, S.” should read -- and Bornemann, S. --.

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"of OXDC" should read -- of OxDC --.

Column 2, Scheme 1 (A)

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